Mechanism of RNA Polymerase Action: Formation of DNA–RNA Hybrids with Single-stranded Templates

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RNA polymerase converts single-stranded DNA templates to a DNA–RNA hybrid. Free RNA does not appear until the composition of the hybrid reaches a limiting value of equal quantities of DNA and RNA. This is in contrast to the situation with double-stranded DNA templates where the double helix is conserved and only free RNA is produced. The bacteriophage ϕX174 DNA–RNA hybrid undergoes a relatively sharp optical density transition with increasing temperature; the T_m of DNA–RNA hybrid is slightly less than that of the corresponding double-stranded ϕX DNA although the dependence of T_m on counter-ion concentration of the two helices is quite similar. After dissociation of the hybrid at high temperature, the helical structure can be reformed by annealing at high ionic strength. The RNA of the hybrid is less susceptible to low levels of pancreatic ribonuclease than the free RNA. ϕX hybrid can serve as template for RNA synthesis; in doing so a major portion of the RNA of the hybrid template is replaced by newly-synthesized RNA; i.e. the reaction is predominantly semiconservative. These results have been discussed in terms of possible models of the copying process.

1. Introduction

DNA polymerase and DNA-dependent RNA polymerase carry out a similar complementary copying of a DNA template (Josse, Kaiser & Kornberg, 1961; Weiss & Nakamoto, 1961). However, the two reactions differ in the fate of the DNA helix which serves as template in the reaction. In the enzymic synthesis of DNA, Wake & Baldwin (1962) have shown, using the dAT‡ copolymer as a model DNA helix, that the strands of the parental DNA separate and are each incorporated into a progeny helix. This is consistent with the semiconservative mode of replication of DNA found *in vivo* by Meselson & Stahl (1958).

In contrast, work in our own and other laboratories has shown that in RNA polymerase catalysed RNA synthesis the template sequences are copied in a fully conservative manner, that is, the template helix remains intact after directing RNA synthesis, and the RNA is found as free RNA (Geiduschek, Nakamoto & Weiss, 1961; Hurwitz, Furth, Anders & Evans, 1962; Chamberlin, Baldwin & Berg, 1963). Since DNA–RNA hybrids can be formed by physical methods (Rich, 1960; Hall & Spiegelman, 1961; Geiduschek *et al.*, 1961), we have examined the enzymic synthesis

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‡ Abbreviations used: dAT = deoxyadenylate-deoxythymidylate copolymer; ϕX DNA = DNA from phage ϕX174; BU = 5-bromouridine; BUTP = 5-bromouridinetriphosphate.

of RNA using single-stranded DNA from ϕX174 as template to determine whether the failure to form a hybrid with helical DNA templates is an inherent feature of the RNA polymerase reaction. We find that the first product of the enzymic reaction using this DNA as template is a DNA–RNA complex; this report concerns the synthesis and some of the properties of this molecule. Enzymic formation of such DNA–RNA complexes with single-stranded templates has also been noted by Warner, Samuels, Abbott & Krakow (1963) and by Sinsheimer & Lawrence (1964).

2. Materials and Methods

Unlabeled ribonucleoside triphosphates and [8-^{14}C]ATP were purchased from Schwarz BioResearch Inc., Orangeburg, New York. [α^{32}P]CTP was prepared by enzymic phosphorylation (Lehman, Bessman, Simms & Kornberg, 1958) of [^{32}P]CMP prepared according to Hurwitz (1959).

E. coli RNA polymerase was fraction 4 enzyme (Chamberlin & Berg, 1962). It was stored frozen in a liquid nitrogen bath in a solution containing 0·1 M-ammonium sulfate, 0·01 M-tris, pH 8, 0·001 M-$MgCl_2$ and 5×10^{-3} M-glutathione. Crystalline pancreatic RNase was purchased from Worthington Biochemical Corporation.

DNA from phage ϕX174 was prepared according to Sinsheimer (1959)† and will be referred to as ϕX DNA. Double-stranded ϕX DNA was prepared as previously described (Chamberlin & Berg, 1962). DNA from λdg phage was prepared after Kaiser & Hogness (1960); DNA from T2 phage was prepared after Josse *et al.* (1961). DNA from calf thymus was prepared after Kay, Simmons & Dounce (1952), and dAT copolymer was synthesized by the procedure of Schachman, Adler, Radding, Lehman & Kornberg (1960). [^{32}P]RNA from *E. coli* was prepared by phenol extraction (Littauer & Eisenberg, 1959) of *E. coli* cells grown in ^{32}P-containing medium (Josse *et al.*, 1961). It was treated with an excess of pancreatic DNase to remove DNA.

Amberlite XE64 resin, equilibrated with sodium citrate buffer, pH 5·4, and 0·05 M-Na^+, was prepared according to Hirs, Moore & Stein (1953). Cesium sulfate was purified as before (Chamberlin *et al.*, 1963). Sephadex G50 was purchased from Pharmacia Fine Chemicals, Ltd., Rochester, Minnesota.

The pH of buffer solutions was determined at 0·05 M concentration unless noted otherwise. Concentrations of nucleic acids are expressed in terms of total nucleotide.

(a) *Preparation of BUTP*

BUTP was prepared by direct bromination of UTP by a modification of a method of Markham (Bessman *et al.*, 1958). 5% bromine in CCl_4 was added dropwise to a solution of 34 μmoles of disodium UTP in 1 ml. of formamide until the bromine color persisted. After 3 min at 25°C, 1 drop of water-saturated phenol was added to remove excess bromine, and 6 ml. of cold ethanol were added to precipitate BUTP. After standing 1 hr at -15°C the solution was centrifuged, and the precipitate was dissolved in 0·01 M-tris buffer, pH 8. This product was then chromatographed on a 10 cm × 1 cm^2 column of Dowex 1(Cl^-, 10% cross-linking), eluting sequentially with 200 ml. of 0·01 M-HCl–0·05 M-NaCl, 80 ml. of 0·1 M-HCl–0·2 M-NaCl, and 80 ml. of 0·5 M-HCl–0·5 M-NaCl. The final solution eluted BUTP which was de-salted by barium precipitation (Lehman *et al.*, 1958). The final product (12 μmoles) had the following spectral ratios in 0·01 M-HCl: 280 mμ/260 mμ = 1·80, 290 mμ/280 mμ = 1·28, 250 mμ/260 mμ = 0·53.

(b) *Preparation of ribosomal RNA*

To 1·8 ml. of a solution of *E. coli* ribosomes (Wood & Berg, 1962) in 5×10^{-3} M-$MgCl_2$ (O.D. at 260 mμ = 150) was added 0·2 ml. of 5% sodium lauryl sulfate. The mixture was incubated 5 min at 37°C, then chilled for 1 hr at 0°C and a precipitate removed by centrifugation. 1 ml. of a 25% suspension of XE64 resin in 0·05 M-sodium citrate, pH 5·4, was

† We wish to thank Dr. R. L. Sinsheimer for helpful discussions, and for his generous hospitality in allowing us to use his facilities for growing ϕX174 phage.

added, then removed by centrifugation. The supernatant solution was diluted to 10 ml. in the same buffer, passed through a 10 cm × 1 cm^2 column of XE64, and the RNA was precipitated by bringing the effluent to 0·5 M in NaCl and 60% in ethanol and dissolved in 1 ml. of 0·05 M-sodium citrate buffer, pH 5·4. RNA prepared by this method showed two sharp boundaries with mean sedimentation coefficients of 18 and 24 s in 0·1 M-NaCl when examined in the analytical ultracentrifuge using u.v. optics.

(c) *Density-gradient centrifugation*

Analytical density-gradient centrifugation in cesium sulfate was carried out essentially as previously described (Inman & Baldwin, 1962*a*,*b*; Wake & Baldwin, 1962). In all experiments 0·45 ml. of a solution containing 44·5% by weight of cesium sulfate in 0·01 M-tris, pH 8 ($\rho = 1{\cdot}547$), 2 mμmoles of dAT copolymer, and 6 to 30 mμmoles of the nucleic acid preparation to be examined, was centrifuged for 12 to 18 hr at 59,780 rev./min and 25·9°C before photographing. All photographs were traced using a Joyce–Loebl double beam recording microdensitometer fitted with a 0 to 2·5 wedge. Densities were calculated relative to dAT which was assigned a density of 1·426, by the method of Ifft, Voet & Vinograd (1961). A value of $1/\beta_0$ of $1{\cdot}685 \times 10^{-9}$ was used.† The buoyant densities observed for bands having a density of less than 1·54 were reproducible to within ± 0·003 g/cc.

Preparative density-gradient centrifugation was carried out in the following manner: 3·0 ml. of 42·8% cesium sulfate ($\rho = 1{\cdot}516$) containing 0·03 M-tris, pH 8, 0·001 M-EDTA, and up to 0·5 μmole of nucleic acid were centrifuged at 33,000 rev./min in the SW39 rotor of the Spinco model L ultracentrifuge, for 3 days. After the centrifugation was stopped, the bottom of the tube was punctured with a needle, and drop fractions were collected. The density of each of the fractions was determined by direct weighing in a calibrated 40 μl. micropipette.

(d) *Synthesis of ϕX hybrid*

The reaction mixture (0·25 to 5 ml.) contained: 40 mM-moles tris buffer, pH 8, 1 mM-$MnCl_2$, 4 mM-$MgCl_2$, 12 mM-2-mercaptoethanol, 1 mM each ATP, UTP, CTP and GTP, 0·6 mM-ϕX DNA, and 200 μg/ml. of fraction 4 RNA polymerase. Synthesis was followed by measuring the amount of radioactivity made acid-precipitable using either [^{14}C]ATP or [^{32}P]CTP as the labeled substrate. After the appropriate time at 37°C the solution was chilled, the nucleoside triphosphates and divalent metal ions were removed either by passage through XE64 resin in 0·05 M-sodium citrate, pH 5·4, followed by dialysis against sodium citrate buffer containing EDTA (Chamberlin *et al.*, 1963) or by passage through Sephadex G50 equilibrated with a solution containing 0·3 M-NaCl–0·01 M-tris, pH 8, and 0·01 M-EDTA.

Characterization of ϕX hybrid was carried out either on the total nucleic acid of a reaction in which the ratio of RNA to DNA was 1 (samples equivalent to the one shown in Fig. 1(e)), or on hybrid isolated from a preparative density gradient. In the latter case, preparations of ϕX hybrid and ϕX RNA were obtained which showed a single component at the expected density when examined in an analytical density gradient. Samples of hybrid obtained by the two methods did not differ detectably in buoyant density or in thermal melting behavior. ϕX hybrid preparations used for thermal melting experiments were handled as previously described to avoid degradation by RNase contamination (Chamberlin *et al.*, 1963).

(e) *General methods*

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Methods used for optical-density melting determinations were as previously described (Inman & Baldwin, 1962*a*,*b*; Wake & Baldwin, 1962).

† These values for $1/\beta_0$ and for the buoyant density of dAT in cesium sulfate containing 0·01 M-tris, pH 8, at 25°C and at 150 atm. pressure were kindly provided by Dr. R. L. Baldwin of Stanford University.

3. Results

(a) *RNA synthesis using ϕX DNA as template*

In the experiments shown in Fig. 1, ϕX DNA was used as a template for RNA synthesis with RNA polymerase and the fate of the DNA template and RNA product was followed by analytical density-gradient centrifugation in cesium sulfate. In the early stages of RNA synthesis directed by ϕX DNA, the free DNA disappears and is replaced by a component having a greater buoyant density (Fig. 1). The density

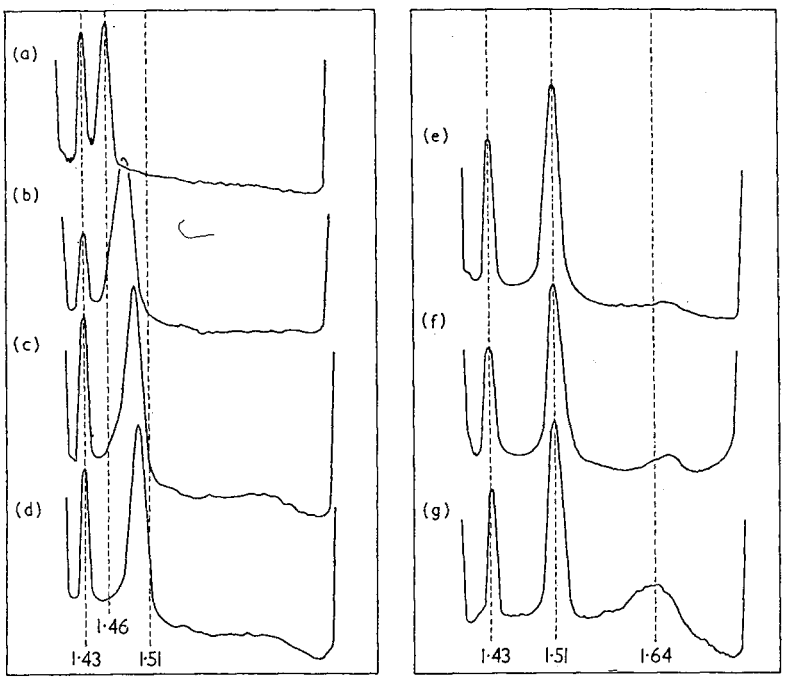

FIG. 1. Stages in the synthesis of ϕX RNA. The figures shown are densitometer tracings from u.v. photographs of a cesium sulfate density-gradient prepared in the analytical ultracentrifuge (see Materials and Methods). In each experiment the cell contained about 2 mμmoles of dAT copolymer as a density marker and about 20 mμmoles of nucleic acid from an RNA polymerase-catalysed synthesis of ϕX RNA. The synthesis was carried out as described in Materials and Methods except that 100 μg/ml. of fraction 4 RNA polymerase was used, and the incubation temperature in experiments 1b, c and d was 25°C. Products were isolated by passage through Sephadex. The length of the enzymic reaction, the ratio of RNA synthesized to DNA primer added, and the buoyant density observed in each experiment are summarized below:

Experiment	Incubation time (min)	$\frac{\text{RNA}}{\text{DNA}}$	Buoyant density of DNA or hybrid band (g/cc)
a	0	0	1·456
b	7 (25°C)	0·3	1·482
c	14 (25°C)	0·5	1·487
d	25 (25°C)	0·7	1·492
e	15	1·0	1·504
f	30	1·1	1·508
g	90	2·0	1·506

of this new component increases continuously until the amount of RNA and DNA in the reaction is equivalent, and then remains constant at 1·50 to 1·51. Further RNA synthesis has no apparent effect on the buoyant density of this new component.

In marked contrast to what is found when helical DNA serves as template, in the early stages of the ϕX DNA-directed synthesis, little of the newly-formed RNA appears as free RNA until the ratio of RNA to DNA is about 1; only then does further synthesis produce appreciable free RNA. This observation, taken with the shift in the density of the DNA, suggests that the RNA remains complexed with the DNA template during synthesis. The data shown here suggest that the ratio of RNA to DNA in the complex increases until it reaches 1 and then remains constant. This complex will henceforth be referred to as ϕX hybrid.

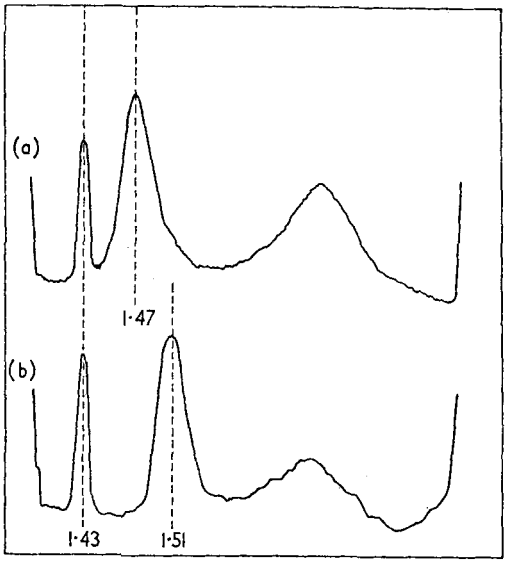

FIG. 2. Attempted hybrid formation from isolated ϕX RNA and ϕX DNA. Densitometer tracings were obtained as described in Fig. 1. (a) 27 mμmoles of ϕX DNA and 25 mμmoles of isolated ϕX RNA were incubated together for 1 hr at 25°C in 0·25 ml. final volume, containing the components described for RNA synthesis but lacking the four ribonucleoside triphosphates. The nucleic acid in the incubation mixture was then isolated by phenol treatment followed by dialysis and subjected to density gradient examination $\rho_{DNA} = 1{\cdot}472$. (b) An incubation was carried out as in (a) but no ϕX RNA was added and the four ribonucleoside triphosphates were included. The products were isolated as in (a). RNA synthesis exceeded the amount of DNA template added by about 1·5 times, $\rho_{Hybrid} = 1{\cdot}506$.

One possible explanation for the origin of the ϕX hybrid is that it is formed non-enzymically during the incubation by association of the template DNA with free RNA formed in the reaction. This seemed improbable because of the observation that no hybrid was formed when ϕX DNA and ϕX RNA were kept at 37°C in 0·1 M-sodium citrate buffer, pH 7·5, for periods up to eight hours. However, a more direct test of this possibility was carried out by incubating ϕX DNA and ϕX RNA with RNA polymerase in the same mixture, and under the same conditions used to prepare hybrid. In the absence of the four ribonucleoside triphosphates no RNA synthesis occurs, and no hybrid is formed (Fig. 2), although the slight increase in the density of the DNA isolated from the mixture may result from some association with free

RNA during the incubation. Under the same conditions but with the four ribonucleoside triphosphates replacing the ϕX RNA, hybrid is formed. Thus the formation of hybrid occurs during the assembly of the complementary RNA.

(b) *Characterization of ϕX hybrid*

(i) *Composition of ϕX hybrid*

To determine directly the limiting composition of ϕX hybrid the hybrid and free ϕX RNA were separated by preparative density gradient centrifugation in cesium sulfate as described above. This separation was carried out on the total nucleic acid of a ϕX DNA-directed synthesis similar to that shown in Fig. 1(g)

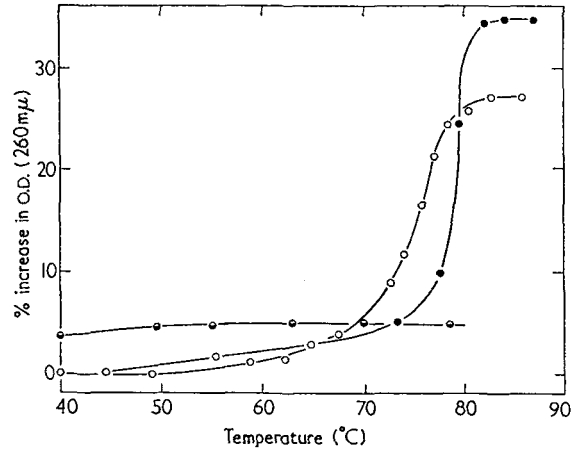

FIG. 3. Optical density melting of ϕX DNA, double-stranded ϕX DNA and ϕX hybrid. The figures show the percentage increase in the optical density at 260 mμ determined at the specified temperature relative to the optical density at 30°C. Melting was carried out in 0·05 M-sodium citrate buffer, pH 7·5 (0·01 M-trisodium citrate, 0·02 M-NaCl; 0·05 M in Na^+). ϕX hybrid was prepared by phenol treatment and dialysis of an RNA polymerase product in which the ratio of RNA to DNA was 1.
—◒——◒— = ϕX DNA; —○——○— = ϕX hybrid; —●——●— = double-stranded ϕX DNA.

in which the ratio of RNA to DNA was 3. The isolated ϕX hybrid contained both RNA and DNA in approximately equal quantities. Thus, the optical density of the ϕX hybrid at 260 mμ after heating and fast-cooling was $15{\cdot}4 \times 10^3$ per mole of RNA nucleotide, while the equivalent value for the isolated ϕX RNA was $8{\cdot}4 \times 10^3$. The RNA content in each case was determined from the [^{32}P]CMP content of the nucleic acid, and the known base composition of ϕX RNA (Chamberlin & Berg, 1962). The extinction coefficient relative to phosphorus of ϕX DNA is $8{\cdot}3 \times 10^3$ (Sinsheimer, 1959), and from this a ratio of RNA to DNA of 1·2 is calculated for the ϕX hybrid.

(ii) *Optical density melting of ϕX hybrid*

When ϕX hybrid is heated in 0·05 M-sodium citrate buffer, pH 7·5, there is a relatively sharp optical density transition, with a T_m of 74 to 75°C (Fig. 3). Single-stranded ϕX DNA under the same conditions shows a broad transition which is essentially complete at 40°C (Sinsheimer, 1959); there is little increase in the optical density in the temperature range between 40 and 80°C. In this behavior single-stranded ϕX DNA resembles single-stranded RNA (Doty, Boedtker, Haselkorn &

Litt, 1959; Cox & Littauer, 1962). Double-stranded ϕX DNA, on the other hand, melts sharply with a T_m of 78·8°C; for comparison one can estimate a T_m of 77°C for a DNA of this base composition from the data of Marmur & Doty (1962). The similarity of the melting curves for ϕX DNA suggests that the hybrid undergoes a temperature induced helix–coil transition.

Although the two optical density transitions are similar, several differences should be pointed out:

(a) the breadth of the temperature transition is greater with ϕX hybrid than with double-stranded ϕX DNA;

(b) the magnitude of the hyperchromic effect is smaller with hybrid ranging from 15 to 25% as compared to 30 to 35% for the double-stranded ϕX DNA, 40 to 45% for naturally occurring double-stranded DNA, and 65 to 70% for double-stranded RNA copolymers (Chamberlin *et al.*, 1963); and

(c) the T_m for the hybrid is lower than that of the double-stranded ϕX DNA at all sodium ion concentrations examined (Fig. 4), although the effect of varying the salt concentration on the T_m is similar for both.

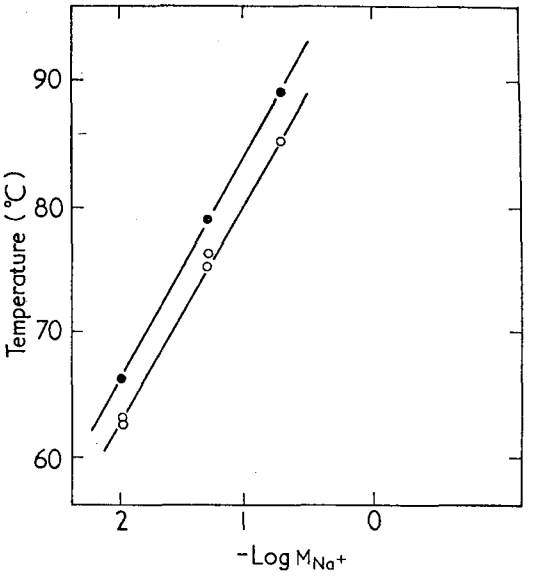

FIG. 4. Variation of the T_m of double-stranded ϕX DNA and ϕX hybrid with the sodium ion concentration. The temperature at the midpoint of the optical density transition (T_m) is shown as a function of the negative logarithm of the sodium ion concentration. Melting was carried out on samples prepared as in Fig. 3 which had been dialysed against a common solution of sodium citrate buffer, pH 7·5, at the sodium ion concentrations shown.
—○——○— = ϕX hybrid; —●——●— = double-stranded ϕX DNA.

The possible significance of these differences will be considered in the Discussion.

(iii) *Strand separation and reassociation with ϕX hybrid*

When a solution of ϕX hybrid is heated through its optical density transition and then cooled to room temperature, the optical density at 260 mμ does not return to its original value. Examination of this denatured hybrid in a cesium sulfate density gradient shows that the hybrid has disappeared and that two new bands have appeared at densities corresponding to free DNA and RNA (Fig. 5(a) and (b)). (The

failure of the DNA band to return completely to its original density is probably due to traces of RNA remaining complexed with it.) Disruption of the ϕX hybrid could also be affected by making the cesium sulfate solution used in the density gradient analysis 0·1 M in NaOH. Under these conditions the RNA is degraded and the hybrid is replaced by a single band having the density of ϕX DNA.

When the thermally separated strands of ϕX RNA and ϕX DNA are incubated for one hour at 60°C in the cesium sulfate solution used for analytical density gradient analysis (1·9 M-cesium sulfate), and then allowed to cool to 30°C over a three-hour period,

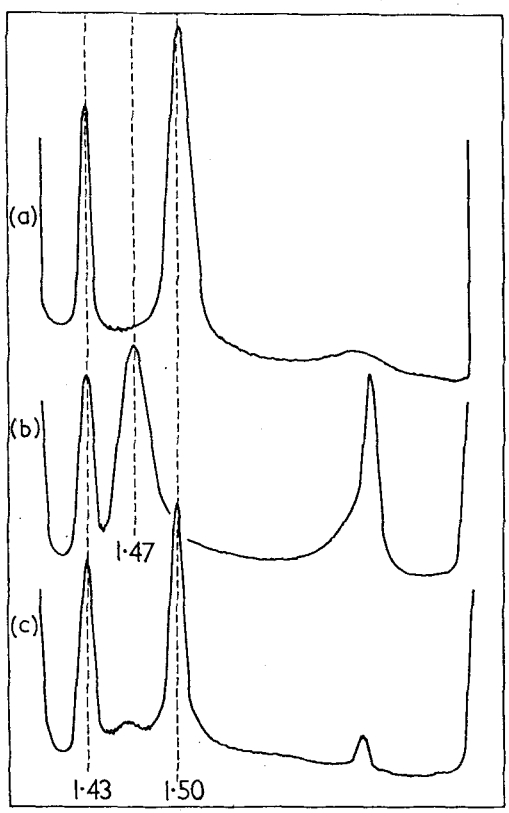

FIG. 5. Strand separation and reassociation with ϕX hybrid. Densitometer tracings were obtained as described in Fig. 1. (a) ϕX hybrid preparation shown in Fig. 1(e), $\rho_{Hybrid} = 1{\cdot}504$ (b) Latter preparation heated 10 min at 85°C in 0·05 M-sodium citrate buffer, pH 7·5, then cooled in an ice bath, $\rho_{DNA} = 1{\cdot}469$, $\rho_{RNA} = 1{\cdot}672$. (c) The ultracentrifuge cell containing the cesium sulfate solution from experiment (b) was heated at 60°C for 1 hr, then cooled to 30°C over a 3 hr period, $\rho_{Hybrid} = 1{\cdot}500$.

the strands reassociate to give a band with the density characteristic of ϕX hybrid (Fig. 5(c)). This reformation of ϕX hybrid can also be demonstrated by annealing a mixture of isolated ϕX DNA and ϕX RNA. Although hybrid reformation occurs under these conditions, we have been unable to reform the hybrid in sodium citrate buffer at sodium ion concentrations up to 0·2 M and minimum conditions necessary for reforming the hybrid from its component parts are not yet known. As an indication of the specificity of hybrid formation under these conditions, no hybrid can be detected ($< 10\%$) when λ DNA or double-stranded ϕX DNA is used in place of single stranded ϕX DNA, or when ribosomal RNA or RNA prepared with T2 DNA as

template replaced the ϕX RNA. RNA made with double-stranded ϕX DNA does *not* form hybrid when heated and slow-cooled in cesium sulfate with double-stranded ϕX DNA (Fig. 6(c))

(iv) *Sensitivity of ϕX hybrid to ribonuclease*

When ϕX hybrid containing [^{14}C]AMP-labeled RNA is incubated with 0·5 to 5 μg/ml. of pancreatic RNase for 15 minutes at 37°C in either 0·1 M-NaCl–0·01 M-tris, pH 8, or in a solution containing 0·01 M-tris, pH 7·3, 0·005 M-$MgCl_2$, 0·007 M-NaCl, and 0·0005 M-EDTA (Yankofsky & Spiegelman, 1962), the radioactivity becomes acid-soluble (Table 1). However, with lower levels of RNase, a differential sensitivity between ϕX hybrid and either ^{32}P-containing *E. coli* ribosomal RNA or

TABLE 1

Ribonuclease sensitivity of ϕX174 hybrid

Component	RNase concentration 0·05 μg/ml. % acid-soluble	0·5 μg/ml. % acid-soluble
E. coli ribosomal RNA	93	> 99
ϕX174 hybrid	18	72
ϕX174 hybrid, heated and quick-cooled	96	98

ϕX hybrid contained RNA labeled with [^{14}C]AMP; *E. coli* RNA, prepared according to Littauer & Eisenberg (1959), was uniformly labeled with ^{32}P. 20 mμmoles of the appropriate RNA were incubated with the amount of RNase shown for 15 min at 37°C in 0·5 ml. of 0·01 M-tris, pH 7·4, 0·10 M-NaCl. The mixture was then assayed to determine the amount of radioactivity still acid-insoluble. Treatment with 5 μg/ml. gave quantitative conversion of the radioactivity from all components to acid-soluble products. ϕX hybrid was isolated from a preparative density gradient centrifugation as described in Materials and Methods.

heat-denatured hybrid is observed. Thus at 0·05 μg/ml. of RNase, 95% of free RNA or RNA of denatured hybrid becomes acid-soluble, while only 18% of the hybrid is degraded. Since it is clear that even at these low levels of RNase significant degradation of hybrid occurs, the use of RNase insensitivity as a test for hybrid or as a preparative technique clearly has some limitations. It should be further noted that RNA alone apparently can exist in RNase-resistant states under certain conditions (Geiduschek, Moohr & Weiss, 1962; Nishimura & Novelli, 1963).

(c) *Hybrid formation with other DNA*

Previous studies have failed to demonstrate formation of a stable hybrid using native DNA templates (Geiduschek *et al.*, 1961; Chamberlin *et al.*, 1963). In confirmation of this, no evidence of hybrid formation is seen when either double-stranded ϕX DNA or native λdg DNA is used as template for RNA synthesis (Figs. 6, 7(a) and (b)). The results with the former DNA exclude the possibility that formation of hybrid with single-stranded ϕX DNA is due to some unique feature of its sequence rather than to its single-stranded character. This is further shown by the fact that when thermally denatured λdg DNA is used to direct RNA synthesis, it is completely converted to a hybrid form (Fig. 7(c) and (d)). This λdg hybrid, like the ϕX hybrid, has a buoyant density in cesium sulfate of about 1·50 to 1·51, and gives rise to free RNA and DNA after thermal denaturation (Fig. 7(e)) and to free DNA on treatment

with 0·1 M-NaOH. Enzymic formation of a hybrid with properties similar to those of *λdg* hybrid is also observed when thermally denatured calf thymus DNA serves as template. It seems reasonable to assume that these hybrids are analogous to ϕX hybrid although confirmation of this must await more detailed characterization of each.

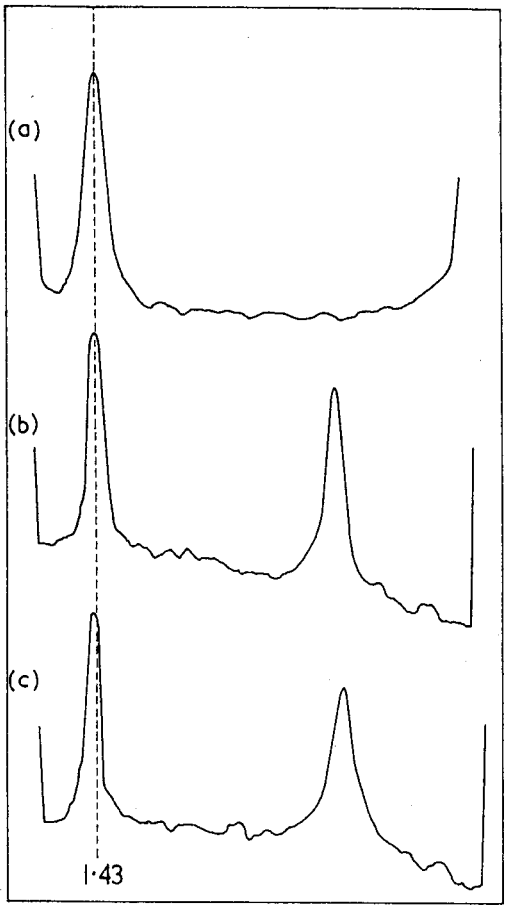

FIG. 6. RNA synthesis with double-stranded ϕX DNA as template. Densitometer tracings were obtained as described in Fig. 1. No dAT copolymer was added as marker in these experiments The figures show: (a) double-stranded ϕX DNA, $\rho = 1{\cdot}432$; (b) total nucleic acid from an RNA polymerase reaction using double-stranded ϕX DNA as template. RNA synthesis was carried out as described for single-stranded ϕX DNA (see Materials and Methods). The reaction was stopped after 60 min when the ratio of RNA to DNA was about 1 and the products were isolated by phenol treatment followed by dialysis; $\rho_{DNA} = 1{\cdot}432$, $\rho_{RNA} = 1{\cdot}650$; (c) the ultracentrifuge cell containing the cesium sulfate solution from experiment (b) was heated at 60°C for 1 hr, then cooled to 30°C over a 3 hr period; $\rho_{DNA} = 1{\cdot}427$, $\rho_{RNA} = 1{\cdot}639$.

Unlike the ϕX hybrid, the *λdg* hybrid did not reform (<10% hybrid formation when the mixture of denatured *λdg* DNA and *λdg* RNA obtained on thermal melting of the *λdg* hybrid was annealed in cesium sulfate as described above. This failure to form hybrid might be due to preferential reformation of the helical DNA structure, the analogous RNA structure, or both. Of importance here may be the observation that the RNA helix has a considerably greater thermal stability than the homologous

DNA helix (Chamberlin *et al.*, 1963; Langridge & Gomatos, 1963) and, by inference, a greater stability than the DNA–RNA hybrid helix. This might contribute to its preferential reformation. It should be noted, however, that Geiduschek *et al.* (1961) have reported the formation of a DNA–RNA complex on annealing denatured T2 DNA with T2 complementary RNA under similar conditions, although the annealing times used were much longer than those employed here.

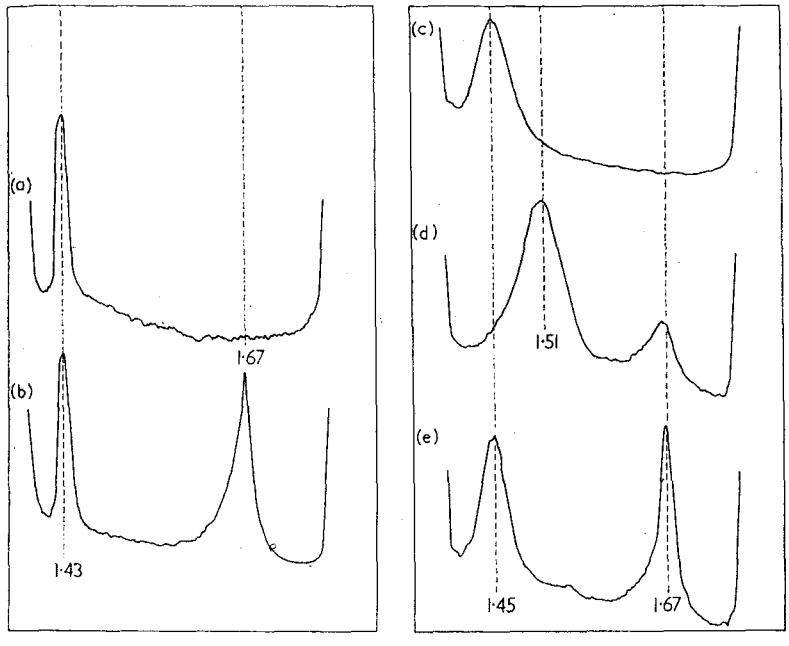

FIG. 7. RNA synthesis using native and denatured *λdg* DNA as template. Densitometer tracings were obtained as described in Fig. 1. RNA products (b) and (d) were synthesized as described for ϕX DNA using a 1 hr incubation and isolated by passage through Sephadex. No dAT copolymer was added as marker in the centrifugation. The figures show: (a) native *λdg* DNA, $\rho_{DNA} = 1{\cdot}426$; (b) total nucleic acid from a reaction in which native *λdg* DNA directed the synthesis of an equal amount of RNA, $\rho_{DNA} = 1{\cdot}426$; (c) denatured *λdg* DNA prepared by heating for 10 min at 100°C in 0·01 M-sodium citrate buffer, pH 7·5, and cooling in an ice bath, $\rho_{DNA} = 1{\cdot}450$; (d) total nucleic acid from a reaction in which heated *λdg* DNA directed the synthesis of a 1·3-fold excess of RNA, $\rho_{Hybrid} = 1{\cdot}509$, $\rho_{RNA} = 1{\cdot}666$; (e) RNA product shown in (d) heated 10 min at 90°C in 0·01 M-sodium citrate buffer, pH 7·5, and then rapidly cooled in an ice bath, $\rho_{DNA} = 1{\cdot}450$, $\rho_{RNA} = 1{\cdot}668$.

Although the experiments described above, together with those previously reported, indicate that a hybrid is not formed when double-stranded DNA is used as primer, the techniques used would not have detected conversion of less than 5% of the DNA to a hybrid form. To establish the maximum amount of hybrid which might be formed with a helical DNA template, we examined the products of a reaction using native *λdg* DNA as template and [α-^{32}P]CTP as the labeled substrate by means of preparative density gradient centrifugation (Fig. 8). The extent of the reaction was limited so that the RNA to DNA ratio was only 0·15. The analysis shows that 80% of the ^{32}P recovered is present at a density characteristic of free RNA, while only 9% of the label is in the density region where DNA–RNA complexes might appear ($\rho = 1{\cdot}40$ to $1{\cdot}52$). Assuming an equivalence of DNA and RNA in any complex

formed, not more than 1·5% of the DNA template remains complexed with the RNA product during the initial stages of RNA synthesis. In view of the difficulty of eliminating the possibility of 1 to 2% of denatured DNA in the λdg DNA preparations used—which would be preferentially converted to hybrid because of the higher affinity of the enzyme for denatured DNA (Hurwitz *et al.*, 1962)—it seems reasonable to assume that a stable DNA–RNA hybrid is not formed as a product of the copying of helical DNA by RNA polymerase.

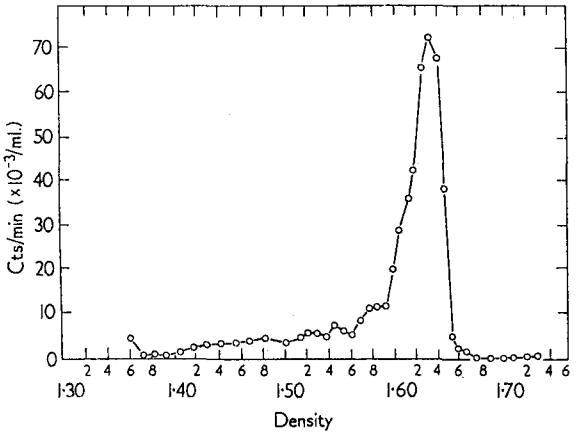

FIG. 8. Preparative density-gradient analysis of RNA synthesis directed by native λdg DNA. 200 mμmoles of λdg DNA were used as template in a reaction mixture similar to that described for the synthesis of ϕX RNA. 33 mμmoles of λdg RNA (2×10^5 cts/min) were produced. The products were isolated by passage through Sephadex and a sample ($3{\cdot}3 \times 10^4$ cts/min) subjected to density gradient centrifugation as described in Fig. 10. The fractions collected contained 65% of the [^{32}P]RNA originally added. The density range from 1·40 to 1·52 ("hybrid") contained 2200 cts/min (9% of the ^{32}P recovered); that from 1·56 to 1·66 (RNA) contained 16,600 cts/min (80% of the ^{32}P recovered).

(d) *RNA synthesis with ϕX hybrid as template*

The fact that ϕX DNA can serve as template for more than a single round of RNA synthesis indicates that the hybrid itself must be able to direct RNA synthesis. One is then led to ask, "Is the hybrid copied conservatively, as is double-stranded DNA, or semi-conservatively as in the case of the DNA polymerase-catalysed replication of double-stranded DNA?" This question was examined by using ϕX hybrid as template in a reaction in which BUTP replaced UTP. As shown in Fig. 9(b), when single-stranded ϕX DNA is copied in the presence of BUTP, a hybrid is produced (bromouracil-containing hybrid) with a greater buoyant density than that of the normal ϕX hybrid in which the RNA contains UMP (uracil-containing hybrid). If the hybrid is copied conservatively, then with a uracil-containing hybrid as template and BUTP as substrate, the hybrid will remain at its starting density and no bromouracil-containing hybrid will be produced. However, if the hybrid is copied semi-conservatively, then the newly synthesized bromouracil-containing RNA should displace the uracil-containing RNA on the hybrid, and after one round of copying, all of the hybrid should band at the bromouracil-containing hybrid density. The data obtained (Fig. 9(d)) indicate that semi-conservative copying does occur. In three separate experiments, an average density of 1·532 was observed for the bromouracil-containing hybrid made from the uracil-containing ϕX hybrid in the

presence of BUTP, as compared to densities of 1·506 and 1·536 found for the uracil- and bromouracil-containing ϕX hybrids, respectively. This corresponds to over 80% replacement of uracil- with bromouracil-containing RNA, if the variation of density with bromouracil-content is assumed to be linear.

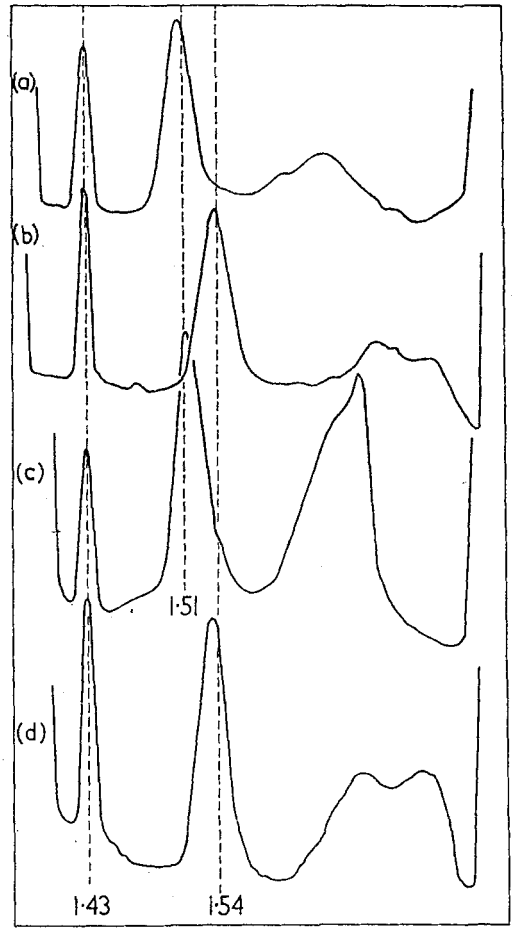

FIG. 9. RNA synthesis using ϕX hybrid as template. Densitometer tracings were obtained as described in Fig. 1. (a) ϕX hybrid, $\rho_{Hybrid} = 1{\cdot}504$; (b) bromouracil-containing ϕX hybrid, $\rho_{Hybrid} = 1{\cdot}535$; (c) total nucleic acid from a reaction in which uracil-containing ϕX hybrid served as template in the presence of UTP, $\rho_{Hybrid} = 1{\cdot}509$; (d) total nucleic from a reaction in which uracil-containing hybrid served as template in the presence of BUTP, $\rho_{Hybrid} = 1{\cdot}532$. The RNA products shown in (a) and (b) were prepared as described in Fig. 1, with a 60 min incubation, except that in (b) BUTP replaced UTP. The ratio of RNA synthesized to DNA template added was 1·5 in each case. Products shown in (c) and (d) were prepared in similar incubation mixtures (0·25 ml.) in which 46 mμmoles of ϕX hybrid with a ratio of RNA to DNA of 1 were used as template. After a 40 min incubation the ratio of newly synthesized RNA to DNA added was about 2. All products were isolated by passage through Sephadex.

A more quantitative evaluation was obtained by using ϕX hybrid containing [^{32}P]RNA as template in an RNA polymerase reaction with unlabeled ribonucleoside triphosphates as substrates, then separating free RNA and hybrid in a preparative density gradient, and determining their ^{32}P content. The results (Fig. 10) show

that most of the ^{32}P-containing RNA appears as free RNA, although a significant fraction (about 30%) still remains in the hybrid. This distribution of ^{32}P between free RNA and hybrid is not significantly changed if bromouracil replaces uracil in the newly formed RNA. When the unlabeled RNA contains uracil the density of the residual ^{32}P in the hybrid peak remains at the hybrid density, as expected; however, when bromouracil replaces uracil in the incoming RNA, the ^{32}P hybrid is shifted to a higher density (Fig. 10(c)). This indicates that all hybrid molecules are copied and

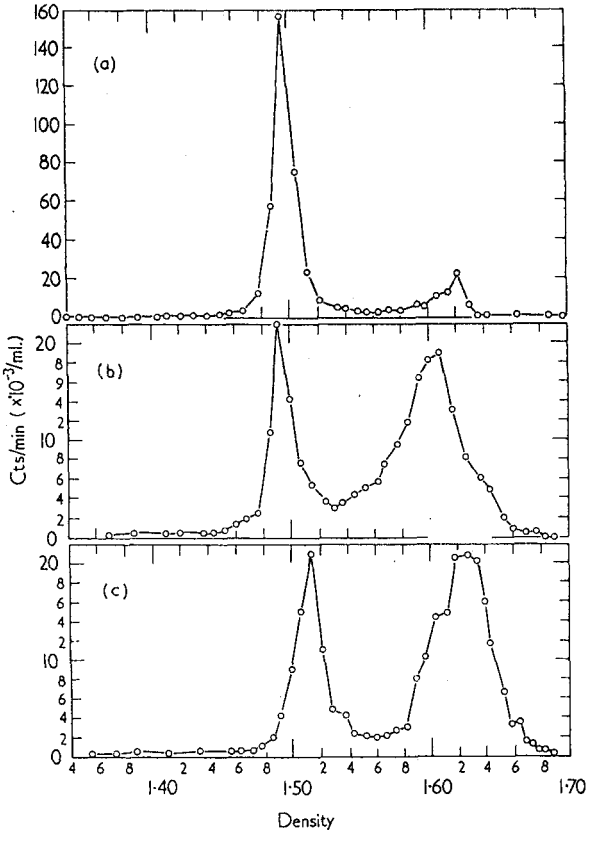

FIG. 10. RNA synthesis using ϕX hybrid as template; preparative density-gradient analysis. The figures show density and ^{32}P content of: (a) fractions obtained from preparative density gradient analysis in cesium sulfate of the [^{32}P]CMP-containing hybrid which was used as template for experiments in Fig. 9(c) and (d). (Ratio of RNA to DNA = 1·1.) ^{32}P added = $3{\cdot}2 \times 10^4$ cts/min; ^{32}P recovered = 64%; (b) fractions obtained from analysis of the total nucleic acid of a reaction in which [^{32}P]CMP hybrid from (a) was used as template in a reaction using unlabeled ribonucleoside triphosphates as substrates. ^{32}P added = $1{\cdot}2 \times 10^4$ cts/min; recovered = 74%. Total ^{32}P in hybrid = $2{\cdot}8 \times 10^3$ cts/min (32%); total ^{32}P in RNA = $6{\cdot}1 \times 10^3$ cts/min (68%); (c) fractions obtained from analysis of the total nucleic acid of a reaction in which [^{32}P]CMP hybrid from (a) was used as template in a reaction using unlabeled ribonucleoside triphosphates and where BUTP replaced UTP. ^{32}P added = $1{\cdot}6 \times 10^4$ cts/min; recovered = 69%. Total ^{32}P in hybrid = $3{\cdot}0 \times 10^3$ cts/min (28%); total ^{32}P in RNA = $8{\cdot}0 \times 10^3$ cts/min (72%).

RNA synthesis was carried out under conditions described in Materials and Methods. In (c) and (d) the ratio of newly synthesized RNA to DNA added was estimated to be 2 from a parallel experiment in which the same hybrid preparation was used as template and [^{32}P]CTP replaced CTP as substrate.

receive heavy, bromouracil-containing RNA, but that certain regions of the hybrid have either not been copied or were copied conservatively.

4. Discussion

To summarize the above observations, RNA polymerase converts single-stranded DNA templates to a DNA–RNA complex. In one such complex, the ϕX hybrid, the amount of RNA is roughly equivalent to the amount of DNA. The thermal melting behavior of this complex suggests that it has a definite helical structure. Finally, the failure of ribosomal RNA or T2 RNA to replace ϕX RNA in the reformation of ϕX hybrid from its components suggests that some sequence complementarity between RNA and DNA exists in the hybrid structure. It is not possible at present to give a clearer picture of the molecular structure of the enzymically formed hybrids, although it is attractive to assume that they have a double helical structure resembling DNA, in which one strand is RNA.

The fact that quantitative differences are noted in the thermally induced optical density transition between double-stranded ϕX DNA and ϕX hybrid raises the possibility that there may be significant structural differences between the DNA–DNA and hybrid helices. The helical structures formed by DNA and RNA by themselves differ appreciably in their dimensions and structures as shown by the differences in their X-ray diffraction patterns (Spencer, Fuller, Wilkins & Brown, 1962; Chamberlin *et al.*, 1963; Langridge & Gomatos, 1963). If these structural differences were poorly accommodated in the hybrid helix, the resulting structure might well have an inherently lower thermal stability, and possibly show a broader optical density transition on melting than either the DNA–DNA or the RNA–RNA helices.

However, alternative explanations of a less fundamental nature are not ruled out by the present data. Differences between the melting of the DNA–DNA helix and the hybrid helix could also result from secondary factors, such as breaks or imperfections in the hybrid structure. Such would be the case if there were appreciable mis-pairing of nucleotide sequences in the hybrid, or if the RNA in the hybrid were of very short chain length. Either of these situations would tend to lower the T_m of the hybrid and broaden the optical density transition (Steiner & Beers, 1961). However, the former possibility is difficult to reconcile with our present understanding of the precision with which RNA polymerase copies the DNA nucleotide sequence (Furth, Hurwitz & Goldmann, 1961; Weiss & Nakamoto, 1961; Chamberlin *et al.*, 1963). With respect to the latter possibility, no appreciable dependence of T_m on chain length is seen in model systems after the molecular weight of the shorter chain exceeds about 30,000 (Lipsett, Heppel & Bradley, 1960; Steiner & Beers, 1961). Although the RNA produced by RNA polymerase with single-stranded templates is significantly smaller than that obtained with helical templates, the mean sedimentation coefficient is still above 4 s (Wood & Berg, 1964) and this suggests that chain length is probably not a critical factor here.

In contrast to the observed formation of hybrids with single-stranded DNA, the above studies exclude the formation of all but trace amounts of hybrid as a stable product in enzymic RNA synthesis with helical templates. Thus, the same DNA nucleotide sequences, which fail to give rise to a DNA–RNA hybrid when copied in their native, helical configuration, do so when copied in single-stranded form. What explanations can be offered for the observed failure of RNA polymerase to produce a hybrid with helical DNA templates?

One model (Stent, 1958; Rich, 1960; Zubay, 1962) would require that the DNA helix remains intact during synthesis, with the DNA hydrogen-bonded *base pairs* specifying the formation of the RNA strand through a new set of hydrogen bonds. We consider this model unlikely, first because it requires different mechanisms of RNA polymerase action when single-stranded and double-stranded DNA are used as templates, and second because it does not account for the displacement of RNA from ϕX hybrid which is found when this structure serves as template.

As an alternative model (Paigen, 1962; Chamberlin *et al.*, 1963) one can picture the attachment of the enzyme to an area of the DNA molecule in which the DNA to RNA base-pairing is temporarily disrupted, possibly at an end. Copying could then proceed along one strand of the DNA as in the case of single-stranded DNA, with transient formation of a region of RNA to DNA base pairs. This region of hybrid helix would then be split in some way to reform the original DNA helix. Two hypotheses will be considered which could account for this reformation. According to the first hypothesis, the nature of the final products would depend solely on the relative stabilities of the possible helical products. Thus, if the DNA helix had a greater stability than the hybrid helix formed, the free DNA strand might spontaneously displace the newly synthesized RNA strand with reformation of the DNA helix. In the case of the DNA polymerase-catalysed copying of DNA, displacement of the newly synthesized DNA strand would not occur, since under these conditions the product of this displacement is the same as the helix which must be split. By this hypothesis the mechanism of both DNA-dependent polymerases would be quite similar with the differences observed being due to the differences in the relative stabilities of the products. As an alternative hypothesis, the enzyme itself might catalyse a displacement of the newly synthesized RNA by the unpaired DNA strand. In this case the displacement could occur even if the hybrid bonds formed were more stable than those of the DNA helix which replaced them. According to this hypothesis there must be fundamental differences in reaction mechanism between RNA polymerase and DNA polymerase.

The experimental data described here do not permit a choice between these alternative explanations for the conservation of the DNA helix. Although the T_m of the ϕX hybrid is lower than the homologous helical DNA (Fig. 4), it is not clear to what extent the thermal stability at low concentrations of sodium ion can be used as a measure of the thermodynamic stability of the helices under the conditions found during synthesis. Moreover, as discussed above, the significance of the difference in T_m between the ϕX hybrid and double-stranded ϕX DNA has yet to be rigorously assessed. In any case, if the difference in stability between the two possible helices were the sole reason for conservative copying of double-stranded templates one would have expected complete turnover of the RNA in the hybrid when the hybrid was used as template. (Assuming, of course, that all regions of the hybrid were copied.) This did not occur. The alternative model, in which the enzyme plays an active role in the reformation of the original template helix would have predicted a conservative mode of copying of the ϕX hybrid. Although this is not what was observed, this possibility cannot be discarded since it may be that enzymic reformation of the original helix occurs less efficiently when one strand is RNA. A choice between the two alternatives may be possible where the potential hybrid can be made to have a greater stability than the template DNA helix itself; such a system is at present under study.

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